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Studies on Methionyl Transfer RNA Synthetase

1. Purification and some Properties of Methionyl Transfer RNA Synthetase from *Escherichia coli* K-12

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Methionyl transferRNA (tRNA) synthetase from *Escherichia coli* strain K-12 was purified 675 fold from extracts of kilogram quantities of cell paste as a product that appears to be homogeneous on chromatography, electrophoresis and ultracentrifugation. The activities measured by the ATP-PP_i exchange assay and by methionyl tRNA formation did not vary significantly during the purification. The final product, which catalyzed the exchange of 1,000 μ moles of pyrophosphate into ATP/mg protein in 15 min at 37°, was obtained with a yield of 50%.

Some general properties of purified methionyl tRNA synthetase are described. The enzyme has a sedimentation coefficient ($s_{20,w}$) of 6.5 S, and a molecular weight of 173,000. In the presence of dissociating reagents (8M urea or 6M guanidine) it undergoes dissociation into subunits which have a molecular weight of about 43,000. Within the limits of the accuracy of the ultracentrifugation analyses, there was no indication of heterogeneity in mass among these subunits. Furthermore, acrylamide gel electrophoresis in the presence of 8M urea failed to reveal any significant heterogeneity of the dissociated enzyme. The results indicate that methionyl tRNA synthetase from *E. coli* K-12 is composed of four, possibly identical, subunits.

In the last few years, an increasingly large number of different aminoacyl transfer RNA (tRNA) synthetases from a variety of sources have been obtained in highly purified form (for a recent review, see Novelli [1]). While studies on these preparations have greatly expanded our knowledge relating to the catalytic functions and other general properties of aminoacyl tRNA synthetases, very little progress has been achieved towards the elucidation of their molecular structure. In particular, no information is yet available on the subunit composition of these enzymes, and on the location of the amino acid and tRNA recognition sites with respect to such subunits.

This investigation of methionyl tRNA synthetase was undertaken with particular emphasis on the problem of subunit composition. In earlier work from this laboratory [2], an apparent molecular weight of 175,000 was assigned to methionyl tRNA synthetase from several strains of *Escherichia coli*, based on elution behaviour during gel-filtration. In the present paper, we describe the purification of the enzyme from *E. coli* K-12, and confirm its molecular weight of 175,000 by sedimentation analysis. Furthermore, evidence is presented indicating that in the presence of 6M guanidine or 8M urea, the enzyme undergoes

dissociation into subunits having an apparent molecular weight of about 43,000. Analysis of the sedimentation patterns, as well as acrylamide gel-electrophoresis in the presence of 8M urea (at one pH value) have failed to reveal any significant heterogeneity of the dissociated enzyme, suggesting that these subunits may be identical. Experiments are presently under way to confirm this possibility.

A forthcoming paper [3] will describe in detail the conditions under which methionyl tRNA synthetase undergoes dissociation into enzymatically active subunits.

During the preparation of this manuscript, two reports dealing with the purification of methionyl tRNA synthetase from *E. coli* strain M. R. E. 600 [4] and *Sarcina lutea* [5], respectively, have appeared in the literature.

EXPERIMENTAL PROCEDURES

MATERIALS

E. coli K-12 cells grown to 3/4 logarithmic phase in minimal medium were ordered from Grain Processing Corp. (Muscatine, Iowa). *E. coli* B tRNA was purchased from General Biochemical Corp. (Chagrin Falls, Ohio). Hydroxylapatite was prepared according to Levin [6]. The Sephadex were products from Pharmacia (Uppsala). L-methioninyl adenylate was

Enzymes. Methionyl tRNA synthetase (L-methionine: sRNA) ligase (AMP) (EC 6.1.1.10); Alcohol dehydrogenase (EC 1.1.1.1).

synthesized by Boissonnas *et al.* [7]. Other chemicals were purchased from the following sources: amino acids and analogues from Mann Research Laboratories (New York), streptomycin sulfate from Specia (Paris), guanidine hydrochloride from Carlo Erba (Milano), ammonium sulfate and glycerol from Merck A.G. (Darmstadt), and [^{14}C]methionine and sodium [^{32}P]pyrophosphate from the Commissariat à l'Energie Atomique (Saclay). The [^{14}C]methionine was further purified as described previously [8].

METHODS

Assay for Amino Acid-Dependent ATP-PP_i Exchange

The activation of methionine was measured by the methionine-dependent ATP-PP_i exchange assay. The reaction mixture contained, in a volume of 1 ml, 100 μmoles Tris-HCl buffer (pH 8.0), 5 μmoles MgCl_2 , 10 μmoles KF, 10 μmoles 2-mercaptoethanol, 2 μmoles ATP (Na salt), 2 μmoles L-methionine, 2 μmoles [^{32}P]PP_i (approximately 150,000 counts/min/assay) and limiting amounts of enzyme. The reaction volume was 0.125 ml. After incubation at 37° for 15 min, the reaction was stopped by the addition of 0.125 ml perchloric acid solution 7% (w/v), followed by 0.4 ml sodium acetate buffer (0.02 M) containing 0.1 M PP_i, pH 4.5 [9], and 0.1 ml of an aqueous suspension of Norit (100 mg/ml). The suspension was thoroughly mixed, then filtered through a Whatman No. 1 paper disc, and the filter washed three times with 10 ml portions of distilled water. The filter was dried under a heat lamp and counted in a thin window gas-flow counter.

One unit of enzyme activity is defined as that amount incorporating 1 μmole of [^{32}P]PP_i into ATP in 15 min at 37°.

Assay for Aminoacyl tRNA Formation

Methionyl tRNA formation was measured as described previously [8]. The reaction mixture contained, in a volume of 0.5 ml, 50 μmoles Tris-HCl buffer (pH 7.4), 5 μmoles MgCl_2 , 5 μmoles KCl, 1 μmole GSH, 1 μmole ATP (Na), 10 nmoles L-[^{14}C]methionine (140,000 counts/min), 1 mg tRNA, and limiting amounts of enzyme. One unit of enzyme is equivalent to the formation of 1 μm of aminoacyl tRNA in 10 min at 30°. The same lot of tRNA was used for assays throughout the purification procedure, since the acceptor capacity was found to vary considerably from one lot to another.

Protein concentration was determined by the method of Lowry *et al.* [10], with crystalline bovine serum albumin as the standard.

Column effluents were monitored for enzyme activity using the ATP-PP_i exchange assay, and for protein by following the absorbance at 280 m μ . The concentration of phosphate buffer in the effluent

fractions was determined on the basis of the refractive index.

Dialysis tubing was freed from impurities by boiling for 15 min in aq. Na_2CO_3 5% (w/v), then rinsing with water, and repeating the procedure using EDTA solution 1% (w/v) at pH 7. The tubing was finally rinsed with water, and stored in a solution of EDTA 0.03% (w/v) at 4°.

Sucrose gradient centrifugation was carried out according to Martin and Ames [11]. 4.4 ml gradients of sucrose from 20 to 5% (w/v) in 0.02 M potassium buffer (pH 7.4) containing 0.01 M 2-mercaptoethanol were used. Purified enzyme (0.5 mg) was layered on top of the gradient and subjected to centrifugation for 20 hours at 3° and 39,000 rev./min, in a Spinco Model L ultracentrifuge. Approximately 25 fractions were collected and assayed for protein content and enzyme activity.

Electrophoresis on polyacrylamide gels, in the absence and in the presence of 8 M urea, was performed as described by Jovin *et al.* [12], using the Tris buffer system. For gels containing 8 M urea, the ionic strength of the Tris-HCl buffer in the lower gel was reduced by half. This resulted in increased mobility of the protein. In the experiment in which riboflavin was used in order to polymerize the lower gel, the ammonium persulfate solution was replaced by an equivalent volume of a solution of riboflavin in 8 M urea solution (1 mg/100 ml). Samples to be submitted to electrophoresis in gels containing 8 M urea were prepared as follows: the purified enzyme (2 mg of protein, recovered by centrifuging a suspension in ammonium sulfate at 80% saturation) was dissolved in 1 ml of 0.19 M Tris-HCl buffer (pH 8.9) containing 8 M urea and 0.1 M 2-mercaptoethanol, and then dialyzed at 25° for 24 hours against 400 ml of the urea solution, with three changes. A portion of the dialyzed solution was immediately used for sedimentation studies in the analytical ultracentrifuge, while aliquots were submitted to gel electrophoresis in 8 M urea.

Analytical gel-filtration through Sephadex G-200 was performed exactly as described in another paper [2]. The purified enzyme (1 mg, dissolved in 1 ml of 0.02 M potassium phosphate buffer, pH 7.4, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol) was loaded onto a column (1.5 $\times$ 75 cm) which was previously calibrated using several marker proteins [2]. Fractions of 0.9 ml were collected at a flow rate of 4 ml/h, and assayed for enzyme activity using the ATP-PP_i exchange reaction, and for protein by the Folin procedure and by absorbance at 280 m μ .

RESULTS

Purification of Methionyl tRNA Synthetase

All operations were carried out at 0–4°. The buffers used throughout the purification procedure were composed of potassium phosphate, and con-

tained 0.01 M 2-mercaptoethanol added just before use. The purification procedure is summarized in Table 1.

Crude Extract. *E. coli* K-12 cells (900 g, wet weight) were suspended in 4,600 ml of 0.02 M phosphate buffer at pH 7.0, and the suspension was subjected to sonic oscillation in a 10 Kilocycle Raytheon oscillator. The lysate was centrifuged at $75,000 \times g$ for 30 min and yielded 4,600 ml of crude extract.

Streptomycin Treatment. Streptomycin (110 g) was slowly added to the extract under constant stirring. The suspension was stirred overnight and then centrifuged at $14,000 \times g$ for 40 min.

at pH 8.1. The column was then washed with 630 ml of 0.1 M phosphate buffer at the same pH, and the enzyme eluted with a linear gradient of phosphate buffer from 0.1 M to 0.25 M at pH 8.1, the total volume of the eluant being 6 liters. The flow-rate was 120 ml/h and fractions of 17 ml were collected. The enzyme peak appeared at about 0.14 M phosphate buffer concentration. The active fractions were combined and dialyzed overnight against 20 liters of 0.02 M phosphate buffer at pH 6.8.

DEAE-Sephadex Chromatography at pH 6.8. The diffusate (970 ml) was loaded unto a column of DEAE-Sephadex (3.2×11 cm) equilibrated with 0.02 M

Table 1. Purification of methionyl tRNA synthetase

Step	Protein	ATP- ³² PP _i exchange assay			Aminoacyl tRNA formation			Ratio of activation vs. charge
		Total activity	Specific activity	Recovery	Total activity	Specific activity	Recovery	
	mg	units	units/mg	%	units	units/mg	%	arbitrary units
Extract	29,800	46,900	1.6	100	427	0.014	100	110
Streptomycin supernatant	—	42,250	—	90	411	—	96	103
Ammonium sulfate fraction, 30 to 50 %	12,150	39,600	3.3	85	346	0.028	81	114
Sephadex G-200 eluate	4,730	35,800	7.6	76	334	0.071	78	107
DEAE-Sephadex pH 8.1 eluate	528	34,375	65	73	333	0.625	78	103
DEAE-Sephadex pH 6.8 eluate	173	27,900	161	60	295	1.71	69	95
Hydroxylapatite pH 6.8 eluate	29	23,400	805	50	235	8.1	55	100
Hydroxylapatite pH 7.5 eluate	21	22,700	1080	48	231	11.0	54	98

Ammonium Sulfate Fractionation. To the streptomycin-supernatant (4,450 ml), solid ammonium sulfate (782 g) was added slowly under stirring, to give 30 % saturation. The mixture was allowed to stand for 1 h with stirring, and then centrifuged for 40 min at $14,000 \times g$. The supernatant fluid was brought to 50 % saturation by the addition of 605 g ammonium sulfate, and the suspension was stirred for 1 h and centrifuged. The precipitate was taken up in 100 ml of 0.02 M phosphate buffer, pH 8.1 and dialyzed overnight against 20 liters of the same buffer. A slight precipitate which appeared during dialysis was discarded after centrifugation at $12,000 \times g$ for 20 min.

Gel-Filtration through Sephadex G-200. The diffusate (240 ml) was subjected to gel-filtration through a column (6.8×97 cm) of Sephadex G-200 (particle size 40–120 μ) equilibrated with 0.02 M phosphate buffer at pH 8.1, and developed with the same buffer. The flow rate was adjusted to 60 ml/h, and fractions of 12 ml were collected and assayed for enzyme activity.

DEAE-Sephadex Chromatography at pH 8.1. The pool of the active fractions (715 ml) was applied directly on a column of DEAE-Sephadex A-50 (5×24 cm) equilibrated with 0.02 M phosphate buffer

phosphate buffer pH 6.8. After washing with 200 ml of the same buffer, containing 0.1 M KCl, the column was eluted with a linear gradient formed from 1.5 liters of 0.1 M KCl and 1.5 liters of 0.3 M KCl, both in 0.02 M phosphate buffer, pH 6.8. Fractions of 14 ml were collected at a flow-rate of 50 ml/h. The enzyme emerged at a KCl concentration of about 0.18 M.

Hydroxylapatite Fractionation at pH 6.8. The pool of the active fractions (520 ml) was directly applied on a column of hydroxylapatite (1.6×9.5 cm) equilibrated with 0.02 M phosphate buffer, pH 6.8. The enzyme was eluted with a linear gradient from 0.02 to 0.15 M phosphate buffer, pH 6.8, the total volume of the eluant being 2 liters. The flow-rate was 40 ml/h and fractions of 10 ml were collected. The activity appeared at a phosphate buffer concentration of about 0.1 M. Those fractions containing the highest specific activity were combined and dialyzed against 10 liters of 0.02 M phosphate buffer, pH 7.5.

Hydroxylapatite Fractionation at pH 7.5. The diffusate (350 ml) was loaded unto a column of hydroxylapatite (1.6×7.5 cm) equilibrated with 0.02 M phosphate buffer, pH 7.5. The column was eluted with a linear gradient from 0.02 to 0.2 M phosphate buffer, the total volume of the eluant being 1 liter.

The flow-rate was 27 ml/h, and fractions of 7.5 ml were collected. The enzyme activity appeared at a phosphate buffer concentration of about 0.06 M, and the specific activity was constant at 1,000 units/mg throughout the peak.

Concentration-Step and Storage. The purified enzyme, in 165 ml, was concentrated by adsorption on a small column of DEAE-Sephadex (1.5×5 cm) equilibrated with 0.02 M phosphate buffer, pH 7.5, followed by continuous elution with 0.2 M phosphate buffer at the same pH. The fractions containing activity were combined (35 ml), and the solution was brought to 70% ammonium sulfate-saturation. The suspension was allowed to stand for 12 h, and was then centrifuged at $75,000 \times g$ for 15 min. The precipitate was dissolved in 3 ml of 0.02 M phosphate buffer, pH 7.5, and dialyzed overnight against 2 liters of the same buffer. The dialyzed enzyme had a ratio of absorption at 280 m μ to that at 260 m μ of 1.80.

After addition of an equal volume of glycerol, the purified enzyme was stored at -15° . The yield as weight of protein in the concentration-step was 86%, and the concentrated enzyme preparation had a specific activity of 1,000 units/mg as measured by the ATP-PP_i exchange reaction, and of 10.8 units/mg when assayed for aminoacyl tRNA formation.

General Remarks on the Purification Procedure. The overall yield of enzyme activity recovered was close to 50%. Counting those active fractions which were deliberately excluded from the column-effluent pools, close to 75% of the initial ATP-PP_i exchange activity present in the crude extract could be accounted for at the end of the purification procedure. The stability of the enzyme is further attested by the fact that aliquots of the crude extract and of the Sephadex G-200 effluent pool, conserved at 0° for the duration of the purification (*i.e.*, 13 days), lost less than 10% of their activity during this interval, when assayed by the ATP-PP_i exchange assay. Furthermore, the activity ratio of the two reactions catalyzed by the enzyme did not vary significantly during the purification (Table 1). Purified enzyme preparations stored in 50% glycerol at -15° lost less than 10% activity in 3 months, and 15 to 20% activity in 6 months.

No evidence for the existence of more than one type of methionyl tRNA synthetase was obtained during the purification. The enzyme in the crude extract conserved at 0° had identical elution properties compared to the purified enzyme, when examined by molecular sieve chromatography on Sephadex G-200 (see below). However, it should be emphasized that incubation of the crude extract at 37° leads to dissociation of the enzyme into active subunits. The characteristics of this dissociation are described in detail in a forthcoming paper [3]. In view of this observation, incubation of the crude extract at 37° for the purpose of degrading the RNA,

i.e., the autolysis step, applied in the purification of several aminoacyl tRNA synthetases [14–16] was inapplicable in the case of methionyl tRNA synthetase. The specific activity of purified methionyl tRNA synthetase from *E. coli* strain K-12 is about twice that reported for the enzyme from strain M.R.E. 600 [4], when assayed by either the ATP-PP_i exchange assay or by aminoacyl tRNA formation.

Criteria of Purity. In addition to the evidence from analytical ultracentrifugation experiments described below, the following observations indicate that the enzyme has been purified to homogeneity:

Re-chromatography of the purified enzyme on hydroxylapatite at pH 7.5, under the conditions used in the purification procedure, as well as analytical gel-filtration on Sephadex G-200 (Fig. 1) gave

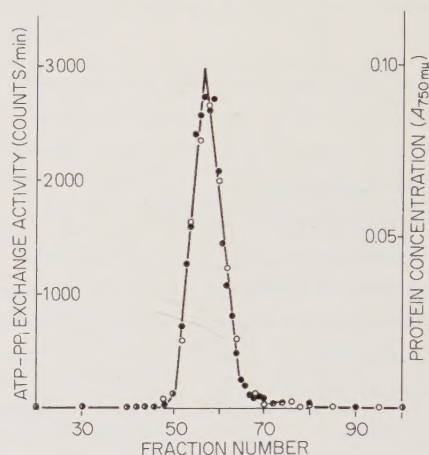


Fig. 1. Gel-filtration behaviour of purified methionyl tRNA synthetase on Sephadex G-200. The enzyme (1 mg) was loaded onto a 1.5×75 cm column, equilibrated and developed with 0.02 M potassium phosphate buffer (pH 7.4) containing 0.1 M KCl and 0.01 M 2-mercaptoethanol. Fractions of 0.9 ml were collected and assayed for enzyme activity using the ATP-PP_i exchange assay (●) and for protein by the Folin procedure (○).

rise to a single, sharp and symmetric peak of enzyme activity, with constant specific activity across the peak. The column used in the gel-filtration experiment was calibrated as described in another paper [2]. Using the empirical calibration method of Andrews, a molecular weight of 174,000 was estimated in excellent agreement with the value found for unfractionated methionyl tRNA synthetase [2].

Sucrose gradient centrifugation yielded a single and sharp activity peak, coinciding completely with the protein peak. The *s* value calculated for methionyl tRNA synthetase was 6.2 S, using yeast alcohol dehydrogenase (7.4 S) as the reference.

Analytical disc electrophoresis of methionyl tRNA synthetase at various stages of purification is shown in Fig. 2. The purified enzyme gave rise to a single band when up to 10 μ g were submitted to electro-

phoresis. Higher loads (up to 80 μg of enzyme) resulted in the appearance of two additional, faster moving components in trace amounts. While these may reflect minor heterogeneity, the possibility that they represent artefacts inherent to this electrophoresis procedure [17] cannot be excluded.

observed reflects the behaviour of the dissociated enzyme. All of the protein penetrated into the lower gel (as assessed by the absence of straining material in the upper gel), and was resolved into a major, a minor and a trace component, in order of decreasing mobility. The staining intensity of the major band

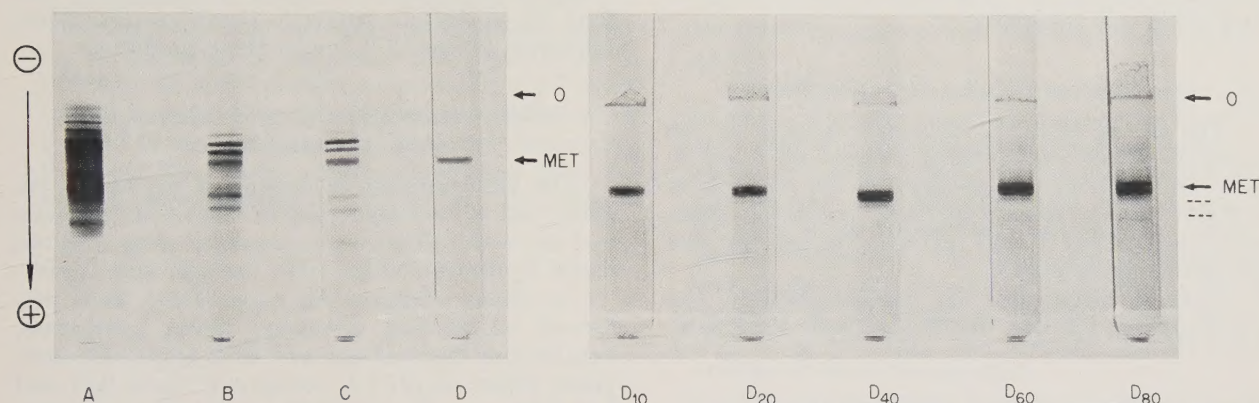


Fig. 2. Polyacrylamide gel electrophoresis using 7.5% standard gels running at pH 8.5 [12]. (A to D): purity at various stages of the purification of methionyl tRNA synthetase. (A) Sephadex G-200; (B) DEAE-Sephadex at pH 8.1; (C) DEAE-Sephadex at pH 6.8; (D) hydroxylapatite at pH 7.5. Conditions: 2 mA per tube for 2 hours at 4°. (D₁₀ to D₈₀): Homogeneity of methionyl tRNA synthetase. The numbers 10 to 80 refer to the amount (in μg) of the purified enzyme, stored in 50% glycerol, which was submitted to electrophoresis. Conditions: 2 mA per tube for 3 hours at 4°. The symbols O and MET designate the origin and the position of methionyl tRNA synthetase, respectively

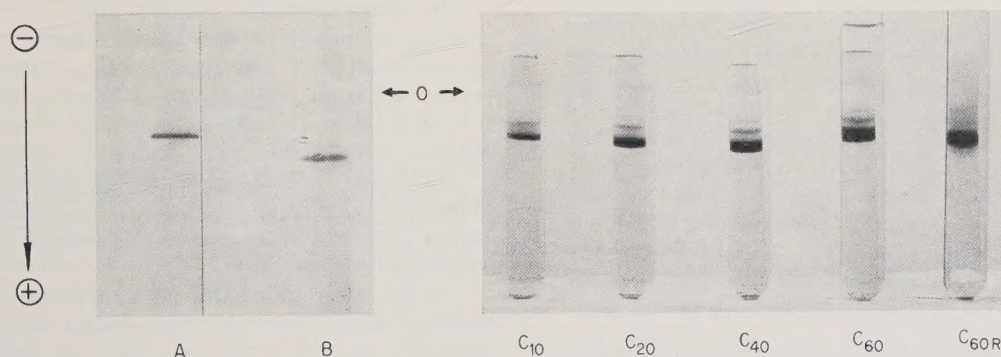


Fig. 3. Polyacrylamide gel electrophoresis using 7.5% standard gels contain 8 M urea, prepared according to [12]. Purified methionyl tRNA synthetase was dialyzed at 25° against Tris-Cl buffer containing 8 M urea, at pH 8.9 (see Methods), and aliquots were submitted to electrophoresis. (A and B): The effect on band mobility of decreasing the ionic strength of the Tris-Cl buffer in the lower gel. (A) full-strength Tris-Cl buffer (*i.e.*, lower gel prepared as in [12]); (B) half-strength Tris-Cl buffer. Approximately 5 μg of protein dialyzed against 8 M urea was run in both gels for 2 h at 4° and 2 mA per tube. (C₁₀ to C₆₀): Band patterns obtained using increasing amounts of the protein dialyzed against 8 M urea. The numbers refer to the amount (in μg) of protein submitted to electrophoresis. C_{60R} represents the band pattern obtained when 60 μg of the protein was applied on a gel polymerized using riboflavin instead of ammonium persulfate, other conditions being identical to C₆₀. Conditions: 2 mA per tube for 3 h at 4°, using half-strength Tris-Cl buffer in the lower gel. The letter O designates the origin

Results of polyacrylamide electrophoresis in 8 M urea at alkaline pH [12] are shown in Fig. 3. An aliquot of the sample submitted to electrophoresis, was simultaneously examined in the ultra-centrifuge and was shown to have undergone dissociation into subunits (see below). Since the composition and the pH of the 8 M urea buffer in which the protein was dissolved was the same as that used in the preparation of the lower gel, it is assumed that the band pattern

was comparable to that observed when the same amount of protein was submitted to electrophoresis in the absence of urea (Fig. 2). The relative intensity of the minor band was variable, ranging from an estimated trace amount to a maximum of 10% of the intensity of the major band. Furthermore, the relative intensity of the minor band was decreased when the same protein sample was examined in gels polymerized in the presence of riboflavin instead of

persulfate (compare C₆₀ to C_{60R}, Fig.3), suggesting that the minor component may arise from oxidation of the denaturated protein by persulfate [13].

The absence of more than one significant component after electrophoresis in 8 M urea argues in favor of the identity of the subunits produced upon dissociation of the enzyme. Experiments are in progress to confirm this possibility.

General Properties of the Purified Enzyme

The rate of ATP-PP_i exchange catalyzed by the purified enzyme was maximal between pH 6 and 9. The same activity was obtained in the presence of 100 mM sodium acetate (pH 6.0), potassium phosphate (pH 7.7) or Tris-Cl (pH 8.5). No activity was detectable below pH 4.0.

Optimal magnesium ion concentration in the exchange reaction was 10 mM. At 5 mM and 2 mM,

Table 2. Comparison of the K_m and K_i values of various substrates and inhibitors of purified methionyl tRNA synthetase in the ATP-PP_i exchange reaction

Substrate	Inhibitor	K_m	K_i
		M	M
L-Methionine	—	4×10^{-5}	—
L-Methionine	L-methioninyl adenylate	—	2×10^{-9}
L-Methionine	DL-methioninol	—	1.5×10^{-5}
L-Ethionine	—	5×10^{-3}	—
L-Norleucine	—	8×10^{-3}	—
ATP	—	3×10^{-4}	—
ATP	L-methioninyl adenylate	—	4×10^{-8}

the rate was 85% and 50% of the maximum, respectively. Methionyl tRNA formation showed a particularly marked dependence on the magnesium ion concentration. In the presence of 2 mg tRNA/ml, optimal activity was observed at 8 mM. Activity was reduced to 50% at 5 mM and 2 mM, and no activity was detectable below 1 mM.

2-Mercaptoethanol, at concentrations up to 10 mM, had no inhibitory effect on the exchange reaction.

The K_m values for methionine, ATP, and two analogs of methionine, ethionine and norleucine, in the ATP-PP_i exchange reaction were determined from Lineweaver-Burk plots. These are summarized in Table 2. Also included in this Table are the K_i values for two competitive inhibitors of the activation reaction, methioninol and methioninyl adenylate [8]. Essentially the same K_m and K_i values were found as those previously reported using partially purified enzyme [8].

D-methionine, tested at concentrations up to 8 mM, and DL-methionine sulfoxide and sulfone, at concentrations up to 20 mM, were completely inactive as substrates in the exchange reaction. Furthermore, no inhibition of the activation of L-methionine (2 mM) was observed in the presence of 2 mM D-methionine and 4 mM DL-methionine sulfone. DL-methionine sulfoxide (4 mM) caused minor inhibition (about 15%) under these conditions.

Sedimentation Behaviour and Determination of the Molecular Weight

The sedimentation behaviour of the native enzyme, and of the enzyme treated with 6 M guanidine and also with 8 M urea, was examined using a Spinco model E ultracentrifuge, with the following results.

(a) *Native Enzyme*. All experiments were performed on purified methionyl tRNA synthetase, exhaustively dialyzed against 0.02 M potassium phosphate buffer at pH 7.4, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol. The protein concentration was estimated from the refractive index of the solutions, assuming a specific refractive increment of 0.185 ml per g. The partial specific volume was not measured. An arbitrary value of 0.74 ml/g was used for the calculations.

In sedimentation velocity runs, the schlieren moving boundary pattern shows a single, sharp and symmetrical peak. The apparent $s_{20,w}^*$ values obtained with 3 different concentrations ranging from 1.5 to 5.0 mg/ml were linearly extrapolated to zero concentration, and yielded an $s_{20,w}^0$ value of $6.45 (\pm 0.06)$.

Evidence of homogeneity of the enzyme preparation is provided by the following tests on the sedimentation pattern obtained with the protein solution at 4.9 mg/ml:

a) the area under the peak, measured by integrating dn/dr (corrected for radial dilution) corresponds to $\Delta n = 92 \pm 2 \times 10^{-5}$, a value which quantitatively accounts for the material introduced into the cell ($\Delta n = 89 \pm 2 \times 10^{-5}$);

b) the analysis of the boundary shape indicates a gaussian distribution. Fig.4 shows a plot of $\delta n_r / \Delta n$, obtained from the data in section (a), vs. the radial distance r inside the cell. This gave a straight line plot within the range of 0.05 to 0.95.

An equilibrium run was performed at 9,945 rev./min and 25° in a short column (0.145 cm) for 22 h. The apparent molecular weight ($\bar{M}_{w,app}$) was calculated from the equation of Lansing and Kraemer [18]. The initial concentration was known, in arbitrary units, from the initial refractive increment (90×10^{-5}) and the optical parameters of the machine. This computed value corresponds to an apparent molecular weight of $165,000 \pm 4,000$. It should be noted that in this and all subsequent molecular weight values, computed using an arbitrary $\bar{v}$ value,

the deviation from the mean reflects the accuracy of the experimental measurements.

Another equilibrium run was performed at a lower protein concentration, using interference optics. A short column (0.174 cm) was used, and the sample was run at a speed of 7,928 rev./min for 22 h at 25°, in a synthetic boundary cell (capillary type). The meniscus fringe concentration was measured according to a method of LaBar (cf. [19] eqn. 6) and, in addition, the initial protein concentration was derived from a boundary-forming experiment performed at the end of the equilibrium run, without dismantling the cell. The initial concentration was 5.1 ± 0.1 fringes, corresponding to 1.3 mg/ml. The plot of

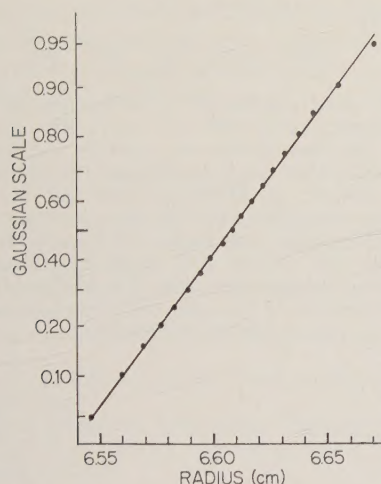


Fig. 4. Velocity sedimentation run of purified methionyl tRNA synthetase. Experimental conditions are described in the text. This gaussian plot of the integral distribution of enzyme within the cell corresponds to a picture taken at 30 min after attainment of the maximum speed (60,000 rev./min)

fringe number versus r^2 gave a straight line, from which the apparent molecular weight of $170,000 \pm 4,000$ was calculated.

A third equilibrium run was performed under the same conditions, at a protein concentration of 0.6 mg/ml. This yielded a $M_{w, app}$ value of $171,000 \pm 7,000$.

In order to estimate the molecular weight at infinite dilution, the reciprocals of the preceding apparent values were plotted against protein concentration. Considering the homogeneity of this enzyme preparation, a molecular weight of $173,000 \pm 7,000$ was assigned to methionyl tRNA synthetase.

(b) *Enzyme Treated with 6 M Guanidine.* All measurements were made in the 6 M guanidine solution described by Ullmann *et al.* [20], prepared by the addition of guanidine hydrochloride to a solution of 0.02 M Tris-Cl buffer pH 7.4, containing 0.01 M EDTA, 0.01 M NaCl and 0.1 M 2-mercaptoethanol.

The native enzyme, recovered by centrifugation of a suspension in ammonium sulfate at 70% saturation, was dissolved in the guanidine solution at a final protein concentration of about 6 mg/ml, and dialyzed against this solution for at least 48 h at room temperature. Appropriate dilutions of the protein solution were then made using fresh guanidine solution, and the resulting samples were dialyzed for an additional 48 h.

The density of the guanidine solution was 1.143 g/ml at 25°. Viscosity values were taken from Kawahara *et al.* [21]. For calculations of molecular weights, an apparent specific volume Φ was used according to Casassa and Eisenberg [22]. This term avoids the problem of the preferential binding of guanidine to protein. Considering the observation of Kirby Hade and Tanford [23], a value of 0.725 ml/g at 20° was assigned to the apparent specific volume Φ (*i. e.*, 0.015 ml/g less than the assumed partial specific volume of the native protein).

For sedimentation velocity experiments, two samples at a concentration of approximately 3 and 6 mg/ml, respectively, were run at 60,000 rev./min and 20°, using schlieren optics. Both samples yielded a single and symmetrical peak. Apparent s_{20}^0 , guanidine values of 0.67 and 0.56 S, respectively, were found at the two protein concentrations. A plot of the reciprocals of these values *vs.* concentration (measured from the area under each schlieren curve) yielded an s_{20}^0 , guanidine value of 0.93 S. Conversion of this value to the water standard gives $s_{20, w}^0 = 2.3$ S, compared with 6.5 S for the native enzyme.

Molecular weight determinations were made by the Archibald approach to equilibrium, using the schlieren optical system. Two protein samples, at a concentration of 3 and 6 mg/ml, respectively, were run at 21,740 rev./min and 25°, with FC 43 to create an artificial bottom. For each run two identical cells were filled to the same level, one with the solution, the other with solvent, in order to account for the redistribution of the guanidine. The apparent molecular weight at the upper meniscus corresponded to $42,000 \pm 2,000$ and $38,000 \pm 2,000$ for the two samples respectively. In both runs, the apparent molecular weights at the two menisci were the same, to the limit of readability of the lower meniscus *i. e.*, until 2 h after the beginning of the run. Extrapolation of the reciprocals of the apparent molecular weights to infinite dilution, using the concentration determined in the preceding velocity runs from the areas under the schlieren curves, gave an $\bar{M}_{w, app}$ value of $45,000 \pm 4,000$.

In order to evaluate the degree of homogeneity of the subunits, a low speed equilibrium sedimentation was performed on another sample, at 5 mg/ml, using a short column (0.19 cm) and interference optics. The run lasted for 20 h at 12,590 rev./min and 20°. The concentration at the upper meniscus was

calculated according to LaBar (cf. [19] eqn. 6a). In addition, the initial concentration was determined at the end of the run by a boundary-forming experiment. A plot of $\ln$ of fringe number (j_r) ($\ln j_r$) vs. r^2 , shown in Fig. 5, yielded a straight line, from the slope of which $\bar{M}_{w, app} = 38,000 \pm 2,000$ was computed.

(c) *Enzyme Treated with 8 M Urea*. A solution of the protein in 8 M urea, at a concentration of about 2 mg/ml, was prepared as already described under the Methods section in connection with the preparation of the sample for electrophoresis on polyacrylamide gels containing 8 M urea.

The density and viscosity values of the 8 M urea solution were taken from Kawahara *et al.* [21]. The

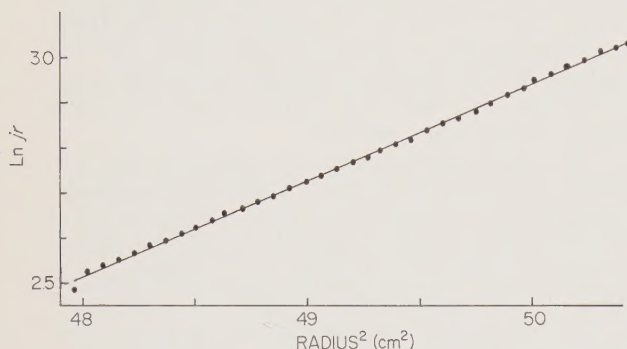


Fig. 5. Low speed equilibrium centrifugation of methionyl tRNA synthetase treated with 6 M-guanidine. The enzyme was dissolved in the guanidine solution to a concentration of 5 mg/ml. The equilibrium sedimentation run lasted for 20 h at 12,590 rev./min. The protein concentration at radius r is expressed in fringe units (j_r). This linear plot ($\ln \cdot j_r/r^2$) was used for the calculation of a w-type apparent molecular weight average

same apparent specific volume (Φ) was used as in the experiments performed in the presence of 6 M guanidine.

A boundary-forming experiment using schlieren optics was carried out on an aliquot of the solution, in order to verify the equimolarity of the urea concentration in the solvent and in the solute. This run also provided, *via* the optical parameters of the machine, the value of the initial protein concentration in terms of fringe units, for use in connection with the low-speed equilibrium run described below.

A low-speed equilibrium run at 12,590 rev./min and 25° for 48 hours, was performed on an aliquot of the preceding solution in a short column (0.26 cm), using interference optics. A plot of the $\ln$ of fringe displacement versus r^2 gave a straight line, from the slope of which $\bar{M}_{w, app} = 43,000 \pm 2,000$ was calculated.

Finally, a high-speed equilibrium run was performed according to Yphantis [24], at a protein concentration of approximately 0.8 mg/ml. The sample was run in a column of 0.65 cm at 33,350 rev./min

and 25° for 47 hours, using interference optics and sapphire windows. Readings of photographic plates were made at 0.1 mm intervals. Fig. 6 shows that the upper meniscus was at zero concentration. From these data, a plot of the $\log_{10}$ of fringe displacement vs. r^2 , shown in Fig. 7, yielded an apparent molecular weight of $41,000 \pm 2,000$.

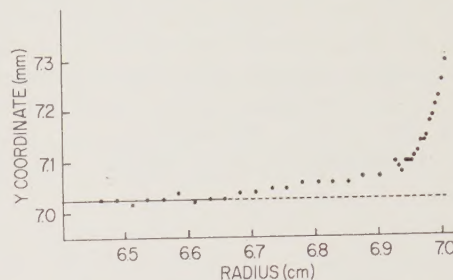


Fig. 6. High speed equilibrium centrifugation of methionyl tRNA synthetase treated with 8 M urea. The enzyme was dissolved in the urea solution at a protein concentration of 0.8 mg/ml and a high speed equilibrium run performed in a column of 0.65 cm at 33,500 rev./min and 25° for 17 h using interference optics and sapphire windows. Readings of photographic plates were made at 0.1 mm intervals. This plot represents the vertical shift of the interference fringes observed on the photographic plate. The horizontal reference base line, indicative of the zero concentration, extends to 2 mm beyond the meniscus

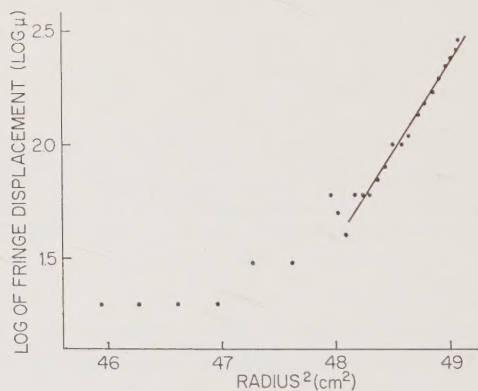


Fig. 7. High speed equilibrium centrifugation of methionyl tRNA synthetase treated with 8 M urea. Log plot of the data from Fig. 4. The concentration is expressed in arbitrary units (μ), one fringe interval being equal to 300 μ . Absolute errors of reading are the same throughout the cell, thus accounting for the poor relative precision of the ten first points. This plot was used to calculate a w-type apparent molecular weight average, corresponding to a mean concentration of 80 μ g/ml. The photographic plate was not readable beyond the last point indicated on the graph

CONCLUSION

A preparation of methionyl tRNA synthetase, pure according to chromatographic and electrophoretic criteria, was examined in the analytical ultracentrifuge in velocity and equilibrium runs, at several speeds and concentrations.

The native enzyme, which behaved as a homogeneous protein under all conditions used, had a sedimentation coefficient ($s_{20,w}^{\circ}$) of 6.5 S, and a molecular weight of 173,000.

In the presence of the dissociating reagents 8 M urea and 6 M guanidine, the enzyme underwent dissociation into subunits. Within the limits of accuracy of the experiments, there was no indication of heterogeneity in mass among the subunits. Their molecular weight corresponds to about 43,000. Furthermore, acrylamide gel electrophoresis in the presence of 8 M urea which was at the same pH and ionic strength as that used in the ultracentrifuge runs, failed to reveal any significant heterogeneity of the dissociated enzyme.

These results indicate that methionyl tRNA synthetase from *E. coli* strain K-12 is composed of four, possibly identical, subunits. Experiments are under way to confirm this possibility.

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